Immunity
Article

Adult Thymus Contains FoxN1^{-} Epithelial Stem Cells that Are Bipotent for Medullary and Cortical Thymic Epithelial Lineages

Ahmet Ucar,[1,2,3,5] Olga Ucar,[1,3] Paula Klug,[1,3] Sonja Matt,[2] Fabian Brunk,[1] Thomas G. Hofmann,[2,4,*] and Bruno Kyewski[1,4,*]
[1]Division of Developmental Immunology, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany
[2]Cellular Senescence Group, German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance, 69120 Heidelberg, Germany
[3]Co-first author
[4]Co-senior author
[5]Present address: Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, M13 9PT Manchester, UK
*Correspondence: t.hofmann@dkfz.de (T.G.H.), b.kyewski@dkfz.de (B.K.)
http://dx.doi.org/10.1016/j.immuni.2014.07.005

SUMMARY

Within the thymus, two major thymic epithelial cell (TEC) subsets—cortical and medullary TECs—provide unique structural and functional niches for T cell development and establishment of central tolerance. Both lineages are believed to originate from a common progenitor cell, yet the cellular and molecular identity of these bipotent TEC progenitors/stem cells remains ill defined. Here we identify rare stromal cells in the murine adult thymus, which under low-attachment conditions formed spheres (termed "thymospheres"). These thymosphere-forming cells (TSFCs) displayed the stemness features of being slow cycling, self-renewing, and bipotent. TSFCs could be significantly enriched based on their distinct surface antigen phenotype. The FoxN1 transcription factor was dispensable for TSFCs maintenance in situ and for commitment to the medullary and cortical TEC lineages. In summary, this study presents the characterization of the adult thymic epithelial stem cells and demonstrates the dispensability of FoxN1 function for their stemness.

INTRODUCTION

T cell development is unique among all hematopoietic lineages; it requires a distinct organ, the thymus. Thymic epithelial cells (TECs) provide unique structural and functional niches, which allow for T cell lineage induction, somatic generation, and subsequent selection ("quality control") of the nascent T cell repertoire (Anderson and Takahama, 2012). The two major subsets of TECs—cortical (cTECs) and medullary TECs (mTECs)—define the two structural compartments of the thymus, the cortex and the medulla. T cells migrate during their development through both compartments in a spatially and temporally ordered process. During the cortical phase, a highly diverse T cell repertoire is generated in a random fashion and subjected to positive selection for self-MHC restriction. The subsequent medullary phase imposes T cell tolerance on the nascent repertoire via negative selection of autoreactive effector cells and differentiation of regulatory T cells (Heng et al., 2010; Manley et al., 2011; Anderson and Takahama, 2012). Failure of developing and/or maintaining an intact and functional thymic epithelial compartment can result either in complete T cell deficiency as exemplified by mutations of the transcription factor FoxN1 or in a skewed T cell repertoire predisposing to autoimmunity as observed in various mutants affecting the NF-κB pathway (Tykocinski et al., 2008; Manley and Condie, 2010).

During mouse embryogenesis, the thymus develops from the third pharyngeal pouch. In mice, thymus development starts around embryonic day 10.5 (E10.5), when parts of the ectodermal cervical vesicle come into close contact with the pharyngeal endoderm. The budding and the outgrowth of the thymic *anlage* occur at E11.5, which is also the onset of *Foxn1* expression in these endodermal cells. The first hematopoietic colonization occurs around E11.5 and the delineation of the cortex and medulla compartments becomes apparent at E14 (Gordon and Manley, 2011). The thymus then increases in size until weaning and after puberty slowly and steadily involutes.

In the postnatal thymus, there is a continuous turnover of TECs. For instance, mature mTECs have a half-life of approximately 2 weeks (Gäbler et al., 2007; Gray et al., 2007; Wang et al., 2012). These observations suggest the existence of self-renewing stem and/or progenitor cells replenishing the mature mTEC subset. Indeed, clonogenic, medullary islet-forming mTEC progenitors have been identified (Rodewald et al., 2001; Hamazaki et al., 2007). In addition, proliferating cTEC progenitors have been characterized in the fetal thymus (Shakib et al., 2009). It is presumed that both lineage-committed precursor pools arise from a bipotent TEC progenitor/stem cell (Bleul et al., 2006; Rossi et al., 2006). Attempts to identify, characterize, and prospectively purify these bipotent TEC progenitor/stem cells have so far met with limited success, and the phenotype of TEC stem cells still remains to be defined (Boehm, 2008; Baik et al., 2013). Yet, the existence of embryonic bipotent TEC progenitors, which could give rise to both cortical and

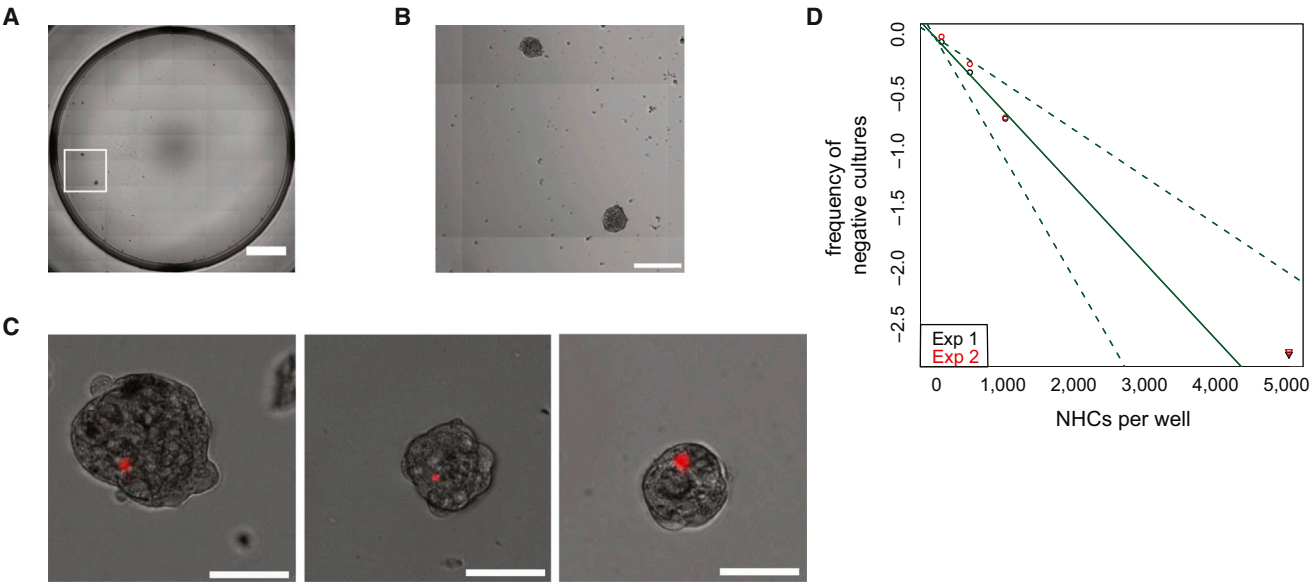

Figure 1. Thymic Stromal Cells Contain Sphere-Forming Cells

(A) Enriched thymic stromal cells isolated from 6-week-old mice formed spheroid structures under low-attachment culture conditions.

(B) Higher magnification of spheres marked by the rectangle in (A).

Scale bars represent 1 mm in (A) and 200 μm in (B). Data are representative of >3 independent experiments.

(C) Thymospheres generated from PKH-26-labeled primary thymic cells contained a single PKH-26^{hi} cell (red staining). Scale bar represents 50 μm. Data are representative of >3 independent experiments.

(D) Extreme limiting dilution assay (ELDA) using thymic digests derived from 5-week-old mice. Exp1 and Exp2 represent two independent experiments. Abbreviation is as follows: NHC, nonhematopoietic cells. Solid line represents the fitted linear regression and the dashed lines demonstrate the 95% confidence interval.

See also Figure S1.

medullary progeny, has been demonstrated in a single-cell transplantation assay (Rossi et al., 2006). In vivo cell lineage tracing revealed the persistence of dormant embryonic TEC progenitors in the postnatal thymus, which still could initiate the formation of a functionally competent minithymus (Bleul et al., 2006). Colony-forming, multipotent thymic cells have also been isolated from the postnatal rat thymus (Bonfanti et al., 2010). However, whether these bipotent progenitors bear the stemness features including self-renewal and low cycling rate remained unclear. Moreover, without the ability to prospectively enrich for thymic epithelial stem cells, the assessment of their developmental potential in vitro or in vivo at the single-cell level has not been possible.

One experimental approach to characterize epithelial stem cell populations ex vivo exploits their ability to form spheroid colonies in the presence of specific growth factors under low-attachment culturing conditions. This method had been first established for neuronal stem cells (neurospheres) (Reynolds and Weiss, 1996) and later been adapted to other tissues of epithelial origin (e.g., of the mammary gland) (Dontu et al., 2003). Under the culture conditions of sphere formation, stem cells maintain the "stemness features" of self-renewal and multipotency. Thus, the sphere-culture method provides a valuable single-cell assay to isolate, characterize, and quantify stem cells with sphere-forming capacity in a species-independent manner (Pastrana et al., 2011).

Here we report that thymic stromal cells of the adult mouse thymus contain rare sphere-forming cells and document that these cells bear the stemness features of low cycling rate, self-renewal, and bipotency, i.e., giving rise to both the cTEC and mTEC lineages, thereby characterizing them as thymic epithelial stem cells. Lineage-tracing experiments demonstrate that these cells originate from the FoxN1^{-} lineage. Moreover, we show that the transcription factor FoxN1 is dispensable for the maintenance and the lineage commitment of these thymic epithelial stem cells.

RESULTS

The Adult Thymic Stroma Contains Sphere-Forming Cells

In analogy to sphere-forming stem cells identified in various other epithelial tissues, we sought to determine whether nonhematopoietic thymic stromal cells also contain a similar cell type. To this end we isolated the thymic stromal cell-enriched fraction from the dissected thymi of adult mice via sequential enzymatic dissociation and subsequent depletion of CD45^{+} cells and cultured them under low attachment conditions in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (see Experimental Procedures). Indeed, spherical structures reminiscent of neurospheres or mammospheres were formed within 7 days in vitro (Figures 1A and 1B). We termed these thymus-derived structures "thymospheres." Of note, the appearance of thymospheres was a slow and progressive process (Figure S1A), suggesting that their formation was not simply due to the aggregation of the cultured cells. Instead, thymosphere formation apparently requires several rounds of cell divisions as observed in other sphere-forming systems.

To verify that thymospheres were derived from a single sphere-forming cell through several rounds of cell division, we performed a label retention assay using the PKH-26-labeling method as described recently (Pece et al., 2010). PKH-26 is a lipophilic membrane dye that integrates into the cytoplasmic membrane of all cell types. During subsequent culturing of the labeled cells in the absence of the dye, the amount of incorporated PKH-26 on cellular membranes will be halved with each cell division. Thus, only slow-cycling cells will retain the PKH-26^{hi} fluorescence. Using this assay it has been shown earlier for mammospheres that at the onset of sphere formation, the sphere-forming cell divides only once in an asymmetric fashion, giving rise to a mitotic bipotent progenitor cell and a quiescent sphere-forming cell (Pece et al., 2010). Other cells subsequently generated during the sphere formation arise from this mitotic progenitor cell and thereby serially dilute the PKH-26 label, whereas only the rarely dividing stem cell retains the PKH-26^{hi} fluorescence. Similarly, using PKH-26-labeled primary thymic stromal cells, we observed that the thymospheres consisted of a single PKH-26^{hi} cell surrounded by a number of PKH-26$^{neg/lo}$ cells (Figure 1C). This result demonstrates that thymospheres formed in these cultures originated from a label-retaining and slow-cycling cell, referred to here as the thymosphere-forming cell (TSFC). Furthermore, this result also rules out the possibility of aggregation as the cause of the observed sphere formation, because aggregation of PKH-26-labeled cultured cells should generate structures containing cells that are equally PKH-26^{hi}, which has never been observed in our thymosphere cultures. Additionally, we performed a mixing experiment where primary thymic cells obtained from two transgenic mouse lines (Rosa-tmTomato and CAG-GFP-tg) were mixed in equal parts and cultured for sphere formation. The presence of GFP-only and Tomato-only thymospheres in these cultures provides additional support for the clonality of the formed thymospheres (Figure S1B).

To determine the frequency of the TSFCs within the isolated primary adult thymic stromal cells, we performed extreme limiting dilution analyses using the ELDA software (Figure 1D; Hu and Smyth, 2009). The TSFC frequency was predicted to be 1 in 1,493 nonhematopoietic cells (NHCs) within the 95% confidence interval of 1/2,405 to 1/926 NHCs. The lack of fit test for single-hit model gave an insignificant p value of 0.196, indicating that a thymosphere is formed by a single cell, i.e., the TSFC. This result also clearly rules out aggregation of the plated cells as an explanation of the observed spheres because this would follow a multihit model. Taken together, our results demonstrated that the adult thymus contains rare sphere-forming cells similar to the ones isolated from various other organs including the mammary gland and the forebrain.

Thymosphere-Forming Cells Have Features of Stemness

Several stemness features distinguish tissue-restricted stem cells from transit-amplifying progenitors and terminally differentiated cells of the corresponding tissue: (1) low cycling rate, (2) self-renewal capacity, and (3) multipotentiality, i.e., the ability to give rise to distinct tissue-specific cell lineages. Accordingly, we asked whether TSFCs bear stemness features and thereby represent thymic epithelial stem cells. First, TSFCs have low mitotic activity as demonstrated in vitro by the PKH-26 label retention assay (Figure 1C). Second, we assessed the self-renewal capacity of the thymosphere-forming cells in vitro by their ability to re-form spheres upon replating of dissociated primary spheres. Thymospheres generated from 5-week-old mouse thymi were size-selected on 35 μm filters (thereby the residual single cells were removed), dissociated into single cells, and recultured, which resulted in the formation of secondary spheres (Figure 2A). Tertiary and quaternary spheres were generated in the same manner in subsequent steps of cultivation, dissociation, and sphere re-formation (Figure 2A). The sphere formation assay has been applied to various epithelial cell lineages as a valuable approach to enrich for stem cells. Indeed, quantification of the TSFC frequency in thymosphere re-formation assay demonstrated a 8.8-fold enrichment of TSFCs upon primary sphere formation (Figure 2B). The TSFC frequency then slightly decreased in the third passage compared to secondary sphere formation as had also been reported for mammospheres (Pece et al., 2010). When thymic stromal cells were only initially labeled with PKH-26 after the dissociation of the thymus and the sphere re-formation assay was repeated (Figure S2), we observed a single PKH-26^{hi} cell in both secondary and tertiary thymospheres (Figures 2C and 2D). This result shows that re-formed spheres were derived from the PKH-26^{hi} label-retaining cells of the previous passage spheres, thus documenting that thymosphere-forming cells indeed have self-renewal capacity.

Next we assessed TSFCs for bipotentiality, i.e., their ability to give rise to both the mTEC and cTEC lineages. Commitment into either mTEC or cTEC lineages was assessed by whole-mount immunostaining of primary thymospheres for cytokeratins 8 and 14 (K8 and K14). Confocal microscopy images demonstrated that thymospheres contained cells expressing either an mTEC lineage marker profile (K14^{+}K8^{-}) or a cTEC lineage profile (K14^{-}K8^{+}) (Figures 3A and S3). The presence of lineage-committed mTECs and cTECs within thymospheres clearly demonstrates the bipotentiality of the TSFCs. Thymospheres also contained cells expressing neither K8 nor K14. We presumed that these cells might represent uncommitted bipotent progenitors. We therefore analyzed the expression of previously described markers of bipotent TEC progenitors: CD205 (also expressed by terminally differentiated cTECs) (Baik et al., 2013), β5t (also expressed by terminally differentiated cTECs) (Ohigashi et al., 2013), and cytokeratin 5 (K5; also expressed by terminally differentiated mTECs) (Klug et al., 1998). Whole-mount coimmunostaining of primary thymospheres for K14 and CD205 (Figure 3B) showed that almost all K14^{-} cells expressed CD205. When we performed K14 immunostaining using primary thymospheres generated from thymic stromal cells isolated from a transgenic mouse line expressing Venus (GFP) under the control of β5t promoter, most of the K14^{-} cells showed β5t promoter activity (Figure 3C). We also generated thymospheres using primary thymic stromal cells isolated from a transgenic mouse line expressing the H2B-GFP fusion protein under the control of K5 promoter. Confocal microscopy analyses of these thymospheres after immunostaining for K8 demonstrated that there were high numbers of K5^{+}K8^{-} cells in these thymospheres (Figure 3D). In addition to these markers, we assessed the expression of epithelial (pan-CK) (Figure 3E), endothelial (CD31) (Figure 3F), and mesenchymal (vimentin) (Figure 3G)

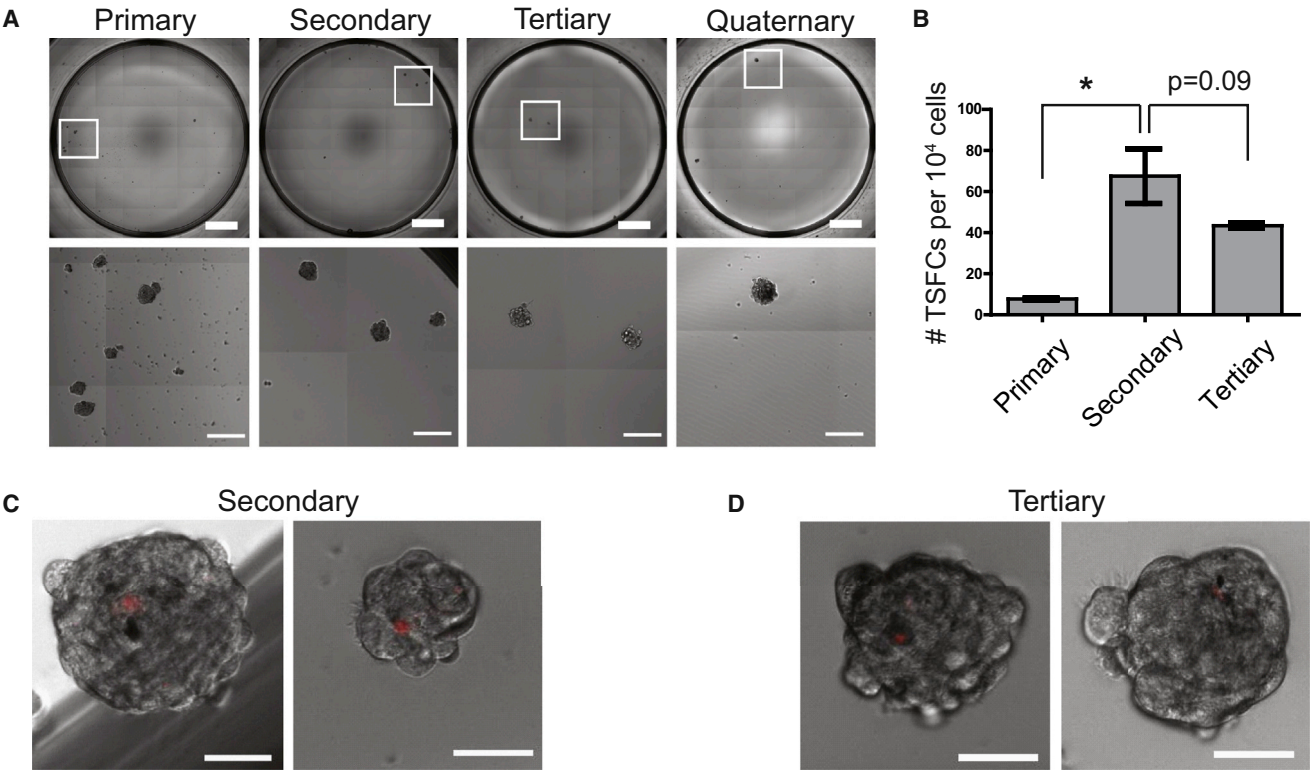

Figure 2. Thymosphere-Forming Cells Are Self Renewing and Slow Cycling

(A) Representative overview (top) and higher-magnification (bottom) pictures of primary, secondary, tertiary, and quaternary thymospheres obtained in sphere re-formation assay. Scale bars represent 1 mm and 200 μm in upper and lower rows, respectively.

(B) Quantification of primary, secondary, and tertiary sphere formation efficiency, shown as the number of spheres derived from 10^4 plated cells (mean ± SEM, n = 3). *$p < 0.05$.

(C and D) Secondary (C) and tertiary (D) thymospheres generated in the sphere re-formation assay using PKH-26-labeled primary thymic stromal cells contain single PKH-26^{hi} cells (red staining). Scale bars represent 50 μm. Representative of two independent experiments.

See also Figure S2.

cell markers within the thymospheres. Expectedly, all cells within the thymospheres were cytokeratin positive, and no CD31- or vimentin-expressing cells were detected in any of the analyzed thymospheres. These data prove the epithelial nature of the thymospheres. Taken together, these results demonstrate that the thymospheres are generated by bipotent thymic epithelial stem cells and are composed of both lineage-committed and -uncommitted thymic epithelial cells. Collectively, our results demonstrate that TSFCs bear all three genuine stemness features and therefore represent adult thymic epithelial stem cells.

Thymospheres Give Rise to Differentiated cTEC and mTEC Lineages In Vitro and In Vivo

To analyze whether the thymic epithelial progenitor cells within thymospheres can generate TECs with their typical morphologies in monolayer cultures, we dissociated the isolated primary thymospheres into single-cell suspensions and cultured them on collagen-coated coverslips. After 9 days of culture, large and flat cells with thymic epithelial morphology were observed, which were either $K14^+$ (mTECs) or $K8^+$ (cTECs) (Figure 4A).

Next, we assessed the potential of thymospheres to form cTECs and mTECs in an in vivo assay. To this end thymospheres derived from genetically marked animals, harboring ubiquitous expression of the Tomato fluorescent protein, were mixed with wild-type cells derived from E14 embryonic thymi in reaggregation thymic organ cultures (RTOCs). After 1 day in vitro culture, reaggregates were transplanted under the kidney capsule of recipient nude mice. At 4–6 weeks after transplantation, the grafts were analyzed by flow cytometry or immunohistochemistry. Flow cytometric analyses confirmed that $Tomato^+$ thymospheres contributed to $EpCAM^+$ TECs within the transplant. A subset of these $EpCAM^+$ donor cells upregulated the maturation marker MHCII, indicative of development into mature TECs (Figure 4B). $Tomato^+$ cells seeded both the cortex and the medulla of the transplant, where they coexpressed cortical (K8) and medullary (K5) lineage markers, respectively (Figures 4C and 4D). Because $UEA1^{hi}$ or $Aire^+$ cells represent the terminally differentiated functional mTECs (Gäbler et al., 2007; Hamazaki et al., 2007), we analyzed the transplants for the expression of these two markers. We were able to detect $Tomato^+UEA1^{hi}$ (Figure 4E) and $Tomato^+Aire^+$ (Figure 4F) cells in the medullary region of the transplant, indicating that sphere-derived TECs were functional and underwent proper terminal differentiation. Taken together, these results demonstrate that the TSFCs and TEC progenitors within the

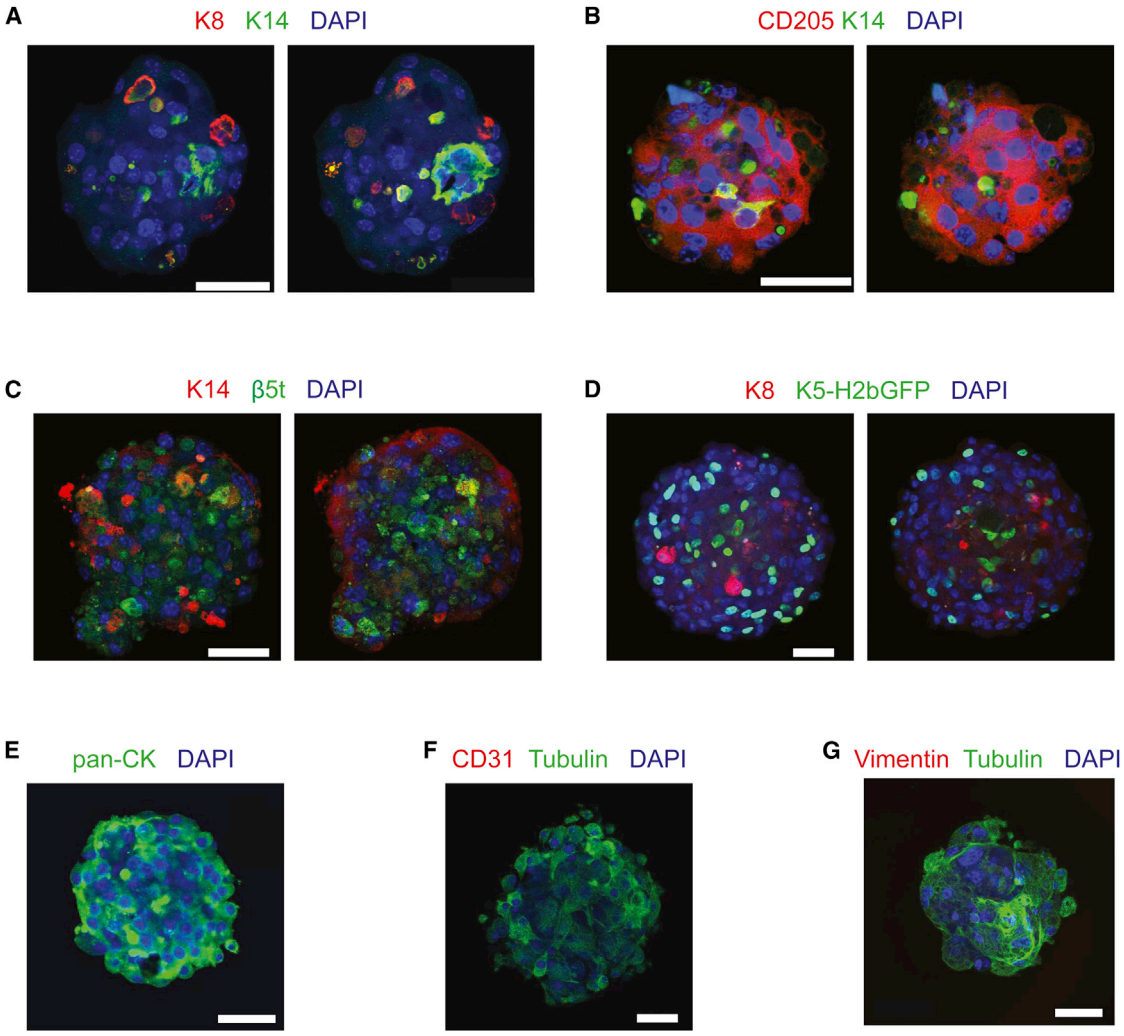

Figure 3. Thymospheres Contain Lineage-Committed mTECs and cTECs and Uncommitted Bipotent TEC Progenitors

(A) Representative confocal image planes of a thymosphere stained for K8 (cTEC marker) and K14 (mTEC marker) expression and counterstained with DAPI.

(B–D) Representative confocal image planes of thymospheres costained for various TEC progenitor markers: CD205 (B), β5T (C), and K5 (D), and K14 or K8.

(E–G) Representative confocal image planes of thymospheres stained for epithelial marker pan-CK (E), endothelial marker CD31 (F), or mesenchymal marker vimentin (G), counterstained with DAPI. Spheres in (F) and (G) were costained for tubulin.

Scale bars represent 40 μm. For all, staining data are representative for ≥10 spheres derived from n ≥ 2 independent experiments. See also Figure S3.

thymospheres can give rise to terminally differentiated medullary and cortical TECs both in vitro and upon long-term reconstitution in vivo.

TSFCs Can Be Enriched Based on Their Distinct Surface Antigen Profile

The low abundance of TSFCs within isolated primary thymic cells prompted us to identify surface markers allowing for their prospective enrichment. To this end, we first fractioned non-hematopoietic cells based on EpCAM expression. Surprisingly, $CD45^{-}EpCAM^{+}$ cells did not form thymospheres, whereas the $CD45^{-}EpCAM^{-}$ cell population contained all TSFCs (Figure 5A). Because not all epithelial cells express EpCAM, but epithelial cell identity is rather defined by cytokeratins (Moll et al., 2008), we assessed cytokeratin expression in primary thymic cells by flow cytometry using a pan-cytokeratin antibody. Indeed, the $CD45^{-}EpCAM^{-}$ cells contained a distinct cytokeratin-positive population (Figure 5B), demonstrating that thymic epithelial cells also reside in the $EpCAM^{-}$ fraction of thymic stromal cells. We next subdivided the $CD45^{-}EpCAM^{-}$ population according to the expression of Sca1 (stem cell antigen 1) and CD24 (also known as heat-stable antigen), both of which have been described as stem cell markers in other tissues (Welm et al., 2002; Shackleton et al., 2006; Stingl et al., 2006). Only the $Sca1^{+}CD24^{-}$ population formed thymospheres, whereas the other populations ($Sca1^{+}CD24^{+}$, $Sca1^{-}CD24^{+}$, and $Sca1^{-}CD24^{-}$) were devoid of TSFCs (Figure 5C). Based on these results we obtained a 4.6-fold enrichment of TSFCs compared to the total thymic stromal cell fraction ($CD45^{-}$ population) via sorting the $CD45^{-}EpCAM^{-}Sca1^{+}CD24^{-}$ subset (Figure 5D).

Next we performed an unbiased surface marker screen (BD, Lyoplate assay) evaluating the expression profile of 176

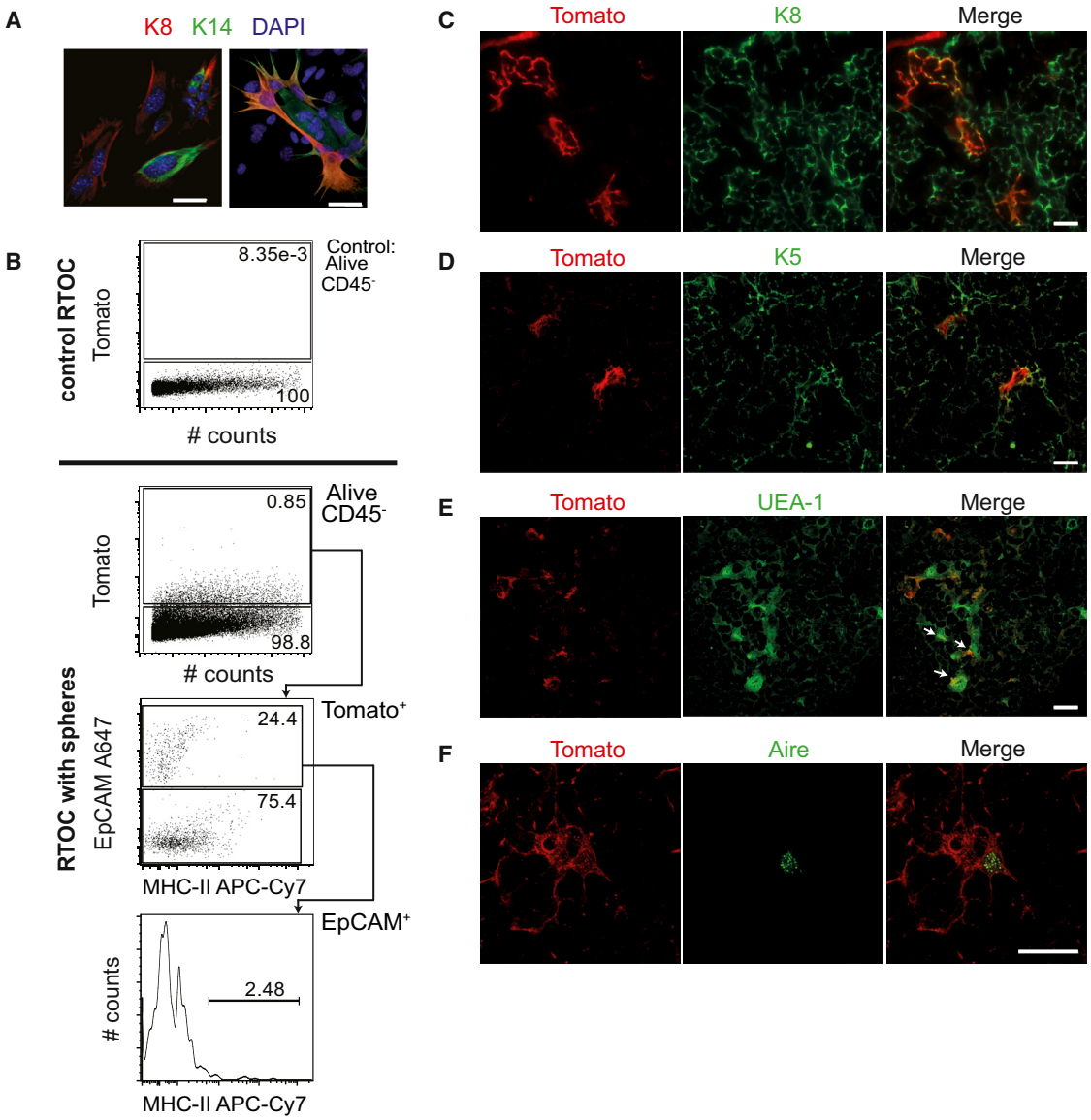

Figure 4. Thymosphere-Forming Cells Give Rise to Both mTECs and cTECs In Vitro and In Vivo

(A) In vitro differentiation in monolayer attachment cultures of cells derived from dissociated thymospheres stained for K8 and K14 expression and counterstained with DAPI (two independent experiments). Scale bars represent 40 μm.

(B) Flow cytometric analysis of RTOCs containing Tomato$^+$ thymospheres 4 weeks after transplantation, assessing EpCAM and MHCII expression by Tomato$^+$ cells. The upper plot represents control transplant, according to which the Tomato gates were set.

(C–F) Histological analyses of transplanted RTOCs containing Tomato$^+$ thymospheres 6 weeks posttransplantation. Sections of transplants were costained for Tomato and either K8 (C), K5 (D), UEA1 (E), or Aire (F). Scale bars represent 50 μm.

Arrows in (E) show the UEA1^{hi}Tomato$^+$ cells.

different surface antigens on pregated CD45$^-$EpCAM$^-$Sca1$^+$ CD24$^-$ stromal cells (Figure S4A). Antigens displaying a relative distribution of 1:3 or more were considered potentially useful for further TSFC enrichment. Based on the outcome of this screen, the CD45$^-$EpCAM$^-$Sca1$^+$CD24$^-$ population was subdivided according to the expression of the surface markers CD38 or CD31. In addition we analyzed the surface marker CD49f, a known stem cell marker in other systems, e.g., mammary gland (Stingl et al., 2006). For all three antigens the positive and negative fractions were assessed for thymosphere formation. For all these markers, the TSFCs clearly partitioned into the respective negative fractions (Figures S4B–S4D). Based on these results, we designed a lineage cocktail (Lin) including biotin-labeled antibodies directed against CD45, EpCAM, CD31, CD38, and CD24. Anti-Ter119 antibody was added to this cocktail in order to exclude erythrocytes. Sorting for Lin$^-$Sca1$^+$ cells resulted in a 6.7-fold enrichment of thymosphere-forming cells over total CD45$^-$ thymic stromal cells (Figures 5E and 5F). This amount of enrichment corresponds to a ratio of 1 TSFC in 296 Lin$^-$Sca1$^+$ cells and thereby allowed

us to perform limiting-dilution experiments resulting in sphere formation even when only 10 Lin$^-$Sca1$^+$ cells were plated per well (data not shown). Taken together, our results demonstrate that TSFCs express a distinct surface antigen profile within the thymic stroma and can be highly enriched as a Lin$^-$Sca1$^+$ cell population.

Thymosphere-Forming Cells Reside within the FoxN1$^-$ Lineage

Expression of the FoxN1 transcription factor in TECs is indispensable for thymus development, because its activity is required for the generation and maintenance of functional mature mTECs and cTECs (Gordon and Manley, 2011). In the absence of a functional FoxN1, e.g., in *nude* mice or FoxN1-deficient mice, thymic development is arrested, which leads to the abolishment of T cell development and thus an immunodeficiency phenotype (Rodewald 2008). Earlier studies suggested that FoxN1 expression in the thymic primordium at E11.25 specifies thymic epithelial identity (Gordon et al., 2001). However, reconstitution of FoxN1 expression in postnatal FoxN1-deficient mice resulted in the development of a functional minithymus, indicating that TEC progenitors were still present in the thymic rudiment of FoxN1-deficient mice even though they were not able to develop into cTECs and mTECs (Bleul et al., 2006). Recent studies provide indirect evidence suggesting that FoxN1 might be dispensable for commitment into the mTEC and cTEC lineages but nevertheless is required for their subsequent differentiation (Nowell et al., 2011). Based on these findings, we asked whether the thymic rudiment of *nude* mice would harbor TSFCs. Indeed, the *nude* thymic rudiment contained TSFCs (Figure 6A), clearly demonstrating that the maintenance of thymic epithelial stem cells does not require the function of FoxN1. Next, we performed lineage-tracing experiments in order to determine whether thymosphere-forming cells reside in the FoxN1$^-$ or FoxN1$^+$ lineage. To this end we generated a double transgenic mouse line, in which FoxN1$^-$ and FoxN1$^+$ lineages were marked by the expression of Tomato and GFP, respectively, whereby GFP expression was indelibly induced by Cre-recombinase expression under the control of the endogenous *Foxn1* promoter (Figure 6B). We assessed thymosphere formation in cells sorted by Tomato versus GFP expression. The TSFCs were found exclusively in the Tomato$^+$ fraction, i.e., in the FoxN1$^-$ lineage, and the FoxN1$^+$ lineage (GFP$^+$) did not form any thymospheres (Figure 6C). *Foxn1* expression was not induced even during sphere formation; all cells within these spheres remained Tomato$^+$ and GFP$^-$ (Figure 6D). As shown above, thymospheres contained lineage-committed cells of both the mTEC and cTEC lineages (Figures 3A and S3). The lack of *Foxn1* upregulation in the sphere culture therefore implies that FoxN1 expression may not be required for mTEC and cTEC lineage commitment. Hence, we analyzed thymospheres derived from the *nude* thymic rudiments by immunostaining for mTEC and cTEC lineage markers and demonstrated that K14$^+$K8$^-$ mTEC and K8$^+$K14$^-$ cTEC lineages emerged in these spheres despite the absence of a functional FoxN1 transcription factor (Figures 6E and S5).

Nevertheless, because FoxN1 is important for the functional development and maintenance of committed TECs, we hypothesized that upregulation of FoxN1 might take place during TEC differentiation after lineage commitment had occurred. Indeed, when spheres derived from FoxN1$^-$ lineage were dissociated and plated under in vitro differentiation culture conditions, we observed a switch from Tomato to GFP expression, indicative of FoxN1 promoter activity (Figure 6F). Taken together, these results document that FoxN1 is dispensable both for the maintenance of the adult thymic epithelial stem cell pool in situ and for the molecular events underlying mTEC and cTEC lineage commitment processes. Therefore, FoxN1 function might be required only for the terminal differentiation and/or survival of TECs.

DISCUSSION

Thymic epithelial cells furnish essential niches for T cell development and for the establishment of central immune tolerance. The cellular and molecular mechanisms underlying early steps in TEC ontogeny and subsequent commitment into both the cTEC and mTEC lineages are still poorly understood. An important missing link in studies on the organogenesis and regeneration of the thymus has been the unambiguous identification and characterization of thymic epithelial stem cells. Several previous studies reported on the identification of early bipotent thymic epithelial progenitors within the thymus of fetal or newborn rodents using different experimental approaches (Bleul et al., 2006; Rossi et al., 2006; Bonfanti et al., 2010). Because these studies focused on bipotentiality but did not further characterize the progenitor cells, which potentially gave rise to both lineages, it remained unclear whether those bipotent cells displayed bona fide stemness features.

Here we isolated thymic epithelial stem cells from the adult thymus on the basis of their sphere-forming potential in ultralow attachment conditions, a conserved feature of stem cells of various epithelial tissues. Next we experimentally assessed the stemness features of these cells. Using PKH-26 labeling, we demonstrate that the thymospheres are formed by a single label-retaining cell, indicating that thymosphere-forming cells are slow cycling, a common feature of tissue-restricted stem cells. Another obligate attribute of stem cells is their self-renewal capacity. Indeed, TSFCs self-renew as shown in vitro by several consecutive rounds of thymosphere dissociation and subsequent sphere reformation. Finally, we document that thymospheres contain cells expressing either an mTEC (K14$^+$K8$^-$) or cTEC (K8$^+$K14$^-$) lineage marker profile. Because all cell types within a thymosphere are descendants of the TSFC, this result indicates that the TSFCs are bipotent. Furthermore, we performed whole-mount immunostaining of thymospheres for various putative markers of bipotent TEC progenitors reported in earlier studies, such as β5t, CD205, and K5. The results demonstrate that thymospheres contain both lineage-committed TECs (K8 or K14 single-positive cells) and uncommitted bipotent TEC progenitors. Additional experiments using in vitro differentiation cultures and transplantation of RTOCs demonstrate that cells derived from thymospheres can further differentiate and generate the corresponding mTECs and cTECs both in vitro and in vivo. The expression of MHCII, Aire, or UEA1 by the transplanted thymosphere-derived cells indicate that the TECs generated from thymospheres are functionally competent in vivo. Hence, TSFCs of adult murine thymi bear three canonical

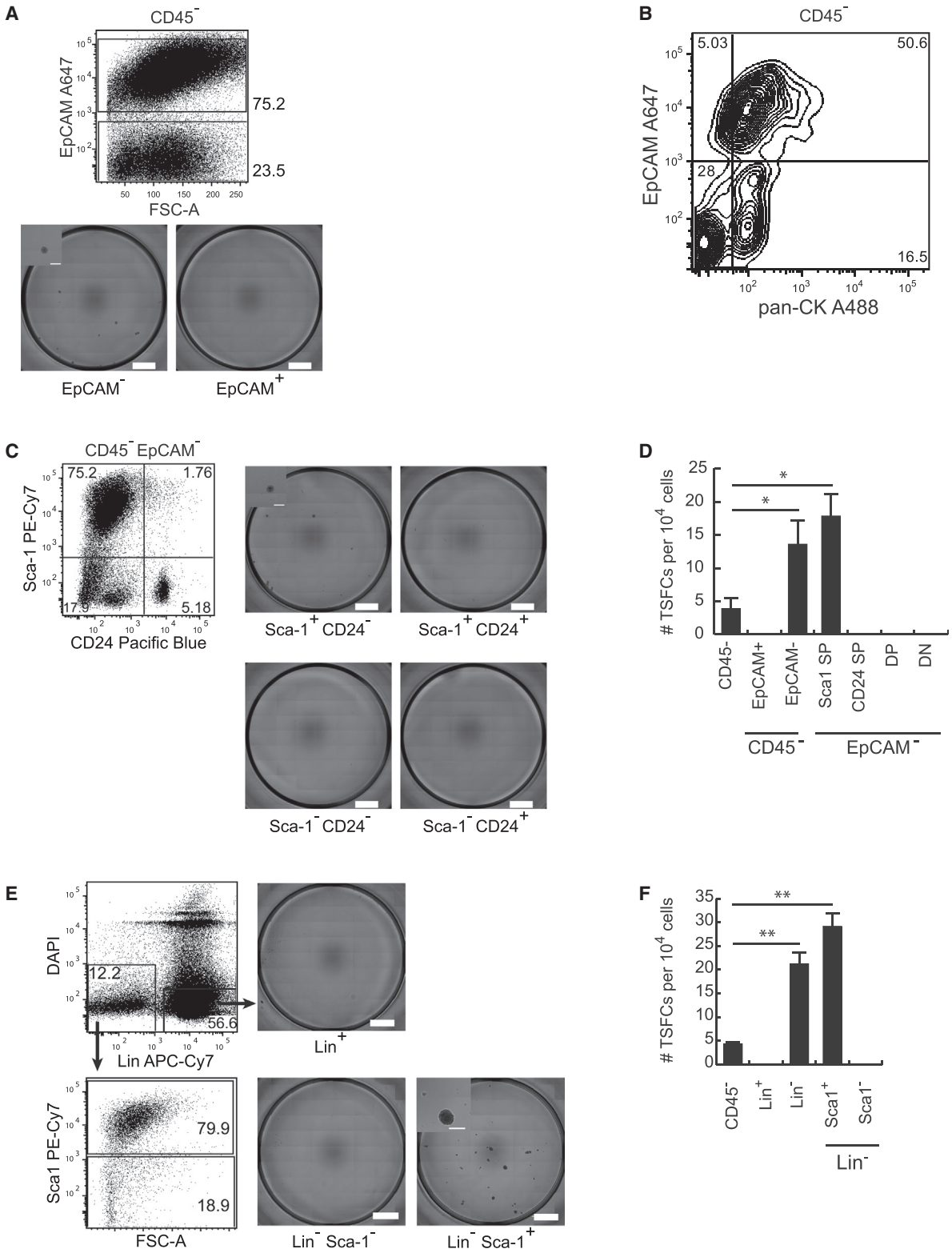

Figure 5. Thymosphere-Forming Cells Can Be Enriched Based on Their Distinct Surface Antigen Profile

(A) Sort windows for CD45$^-$ thymic stromal cells separated on the basis of EpCAM expression and representative pictures of thymosphere cultures of the respective sorted fractions are shown. Inset shows a thymosphere at higher magnification.

(B) Flow cytometric analysis of cytokeratin expression in CD45$^-$ cells. Gates were set based on isotype control staining.

(legend continued on next page)

hallmarks of stemness and thereby qualify as thymic epithelial stem cells.

Using thymosphere formation as a readout, we determined the surface antigen profile of these thymic epithelial stem cells as Lin$^-$Sca1$^+$, whereby Lin refers to a lineage cocktail of antigens including CD45, Ter119, EpCAM, CD24, CD31, and CD38. Importantly, during the identification of the surface antigen profile of TSFCs, we applied stringent selection criteria in order to determine those subpopulations that exclusively contained TSFCs. Based on this surface antigen profile, these rare TEC stem cells can now be highly enriched from dissociated thymic stromal cells of adult mice. Of note, we cannot rule out the possible existence of another epithelial stem cell pool within the adult thymus that does not form thymospheres under our culturing conditions. In such cases, the determined surface antigen profile presented in this study may refer only to the sphere-forming thymic epithelial stem cells.

The finding that TSFCs reside exclusively in the EpCAM$^-$ fraction of the thymic stroma was somewhat surprising because EpCAM expression is commonly considered to be the distinguishing marker of epithelial cells within the thymus. However, EpCAM$^-$ epithelial cells have been described in other tissues (e.g., differentiated keratinocytes) (Trzpis et al., 2007). Moreover, EpCAM expression has been also detected in nonepithelial cells, such as thymocytes and ES cells, not yet committed to epithelial lineage (Trzpis et al., 2007), which suggests that EpCAM expression cannot be considered as a bona fide marker of epithelial cell identity. In contrast, cytokeratin expression unambiguously marks epithelial identity because all epithelial cells express a pair of acidic and basic cytokeratins (Moll et al., 2008). In this respect, cytokeratin expression by a subset of EpCAM$^-$ cells suggests that there are indeed EpCAM$^-$ epithelial cells within the thymic stroma. This finding calls for a re-evaluation of EpCAM as an appropriate TEC marker, at least as far as stem/progenitor cells are concerned. Of note, epithelial stem cells of the mammary gland were also shown to be EpCAM$^{neg/lo}$ (Eirew et al., 2008; Lim et al., 2009) in analogy to our findings.

FoxN1 is a transcription factor that is indispensable for the generation of a functional thymic epithelial compartment. Indeed, the loss of function of FoxN1, as first observed in *nude* mice, results in thymic aplasia and consequent T cell deficiency (Nehls et al., 1994; Rodewald, 2008). However, the precise function of FoxN1 during TEC ontogeny is not exactly understood. Recent studies provided indirect evidence for FoxN1 being dispensable for the maintenance of embryonic thymic epithelial stem cells and lineage commitment into either mTECs or cTECs (Bleul et al., 2006; Nowell et al., 2011). Instead, FoxN1 is apparently required for transition of already committed TECs from early to late differentiation stages and the maintenance of the postnatal TEC compartment (Nowell et al., 2011; Chen et al., 2009; Cheng et al., 2010). We readdressed the function of FoxN1 in the context of sphere-forming adult thymic epithelial stem cells. It was previously suggested that the thymic rudiment of *nude* mice still contains putative thymic epithelial stem cells, because stochastic activation of a functional *Foxn1* gene postnatally led to the development of a fully competent minithymi, thus reverting at least in part thymic atrophy (Bleul et al., 2006). As a direct proof of this claim, we show that the thymic rudiment of *nude* mice does contain sphere-forming thymic epithelial stem cells. Interestingly, as analyzed by lineage tracing, adult thymosphere-forming cells are exclusively present within the FoxN1$^-$ lineage, thus indicating that *Foxn1* is not yet expressed in stem cells but only at later stages. This finding concurs with the presence of thymic epithelial stem cells within the thymic rudiment of *nude* mice, because the loss of function of FoxN1 would not directly affect the stem cell compartment. Importantly, although *Foxn1* was not upregulated in any cell of thymospheres, commitment into mTEC and cTEC lineages did take place within spheres. Furthermore, mTEC and cTEC lineage commitment similarly took place in FoxN1 mutant (*nude*) thymospheres. These results clearly demonstrate that FoxN1 function is dispensable for lineage commitment processes. We observed *Foxn1* promoter activity only during further differentiation in in vitro differentiation culture of dissociated thymospheres, in line with a role of FoxN1 during later stages of TEC differentiation. Taken together, our results document that FoxN1 function is dispensable both for the maintenance of adult thymic epithelial stem cells and their lineage commitment into mTEC and cTEC progenitors.

Although in this study we focused on the functional characterization of TSFCs within the young adult thymus, similar sphere-forming cells exist in the embryonic thymus as well as in the involuted thymus of 18-month-old mice. The comparative quantification of sphere frequency (Figure S1C) demonstrated that the ratio of TSFCs is significantly higher in the embryonic than in postnatal thymus. The relative frequency of TSFCs did not significantly change between young adult and involuted thymus. In this respect, it will be of considerable interest to compare the phenotype and function of TSFCs isolated from a wide range of different age groups in order to address open issues related to embryonic development of the thymus versus adult TEC maintenance and mechanisms underlying age-related thymic involution. Similar questions can also be addressed in humans, because thymosphere-forming cells also exist in the postnatal human thymus (Figure S1D). This will be of particular interest from a translational point of view, because cellular replacement therapies for the treatment of immunodeficiency or autoimmune diseases associated with a dysfunctional thymus would be highly desirable. In this respect, the clonal thymosphere method might present an attractive avenue to enrich for human thymic epithelial stem cells.

(C) Sort windows for CD45$^-$EpCAM$^-$ cells separated on the basis of CD24 and Sca-1 coexpression, and representative pictures of thymosphere cultures of the respective sorted fractions are shown. Insets show thymospheres at higher magnification as formed by the respective subset.
(D) Quantification of thymosphere-forming cell enrichment of the corresponding CD45$^-$ stromal cell subsets are shown (mean ± SEM, n = 3). *$p < 0.05$.
(E) Sort windows for Lin cocktail/Sca-1 coexpression and representative pictures of thymosphere cultures of the respective sorted fractions are shown. Insets show thymospheres at higher magnification as formed by the respective subset.
(F) Quantification of thymosphere-forming cell enrichment of the corresponding cell fractions are shown (mean ± SEM, n = 3). **$p < 0.01$.
Scale bars represent 1 mm for overview and 200 μm for inset pictures. See also Figure S4.

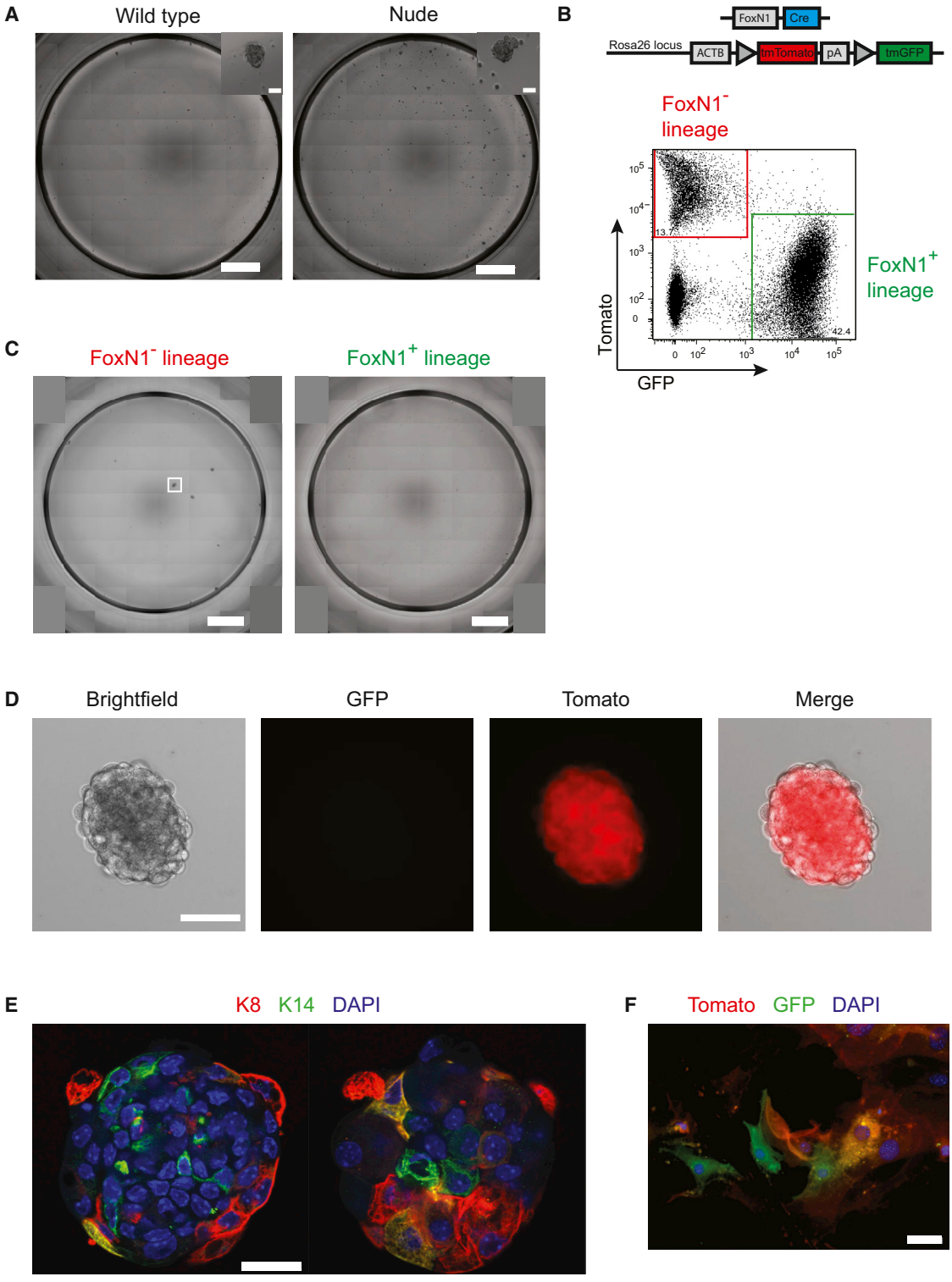

Figure 6. FoxN1 Function Is Dispensable in Thymic Epithelial Stem Cells and Commitment into mTEC and cTEC Lineages

(A) Representative pictures of thymospheres formed by the enriched thymic stromal cells from thymi of NMRI wild-type and *nude* mice. Insets show picture of thymospheres formed by corresponding thymic stromal cells at higher magnification. Scale bars represent 1 mm for overview and 200 μm for inset pictures. Data are representative of two independent experiments.

(B) Sort windows used to purify Tomato^{+} (FoxN1^{-} lineage) and GFP^{+} (FoxN1^{+} lineage) cells derived from Rosa-Tomato-GFP × *Foxn1*^{Cre} double-transgenic animals.

(legend continued on next page)

EXPERIMENTAL PROCEDURES

Animals and Human Material

When not indicated otherwise, 4- to 6-week-old wild-type C57BL6/N mice were used for the isolation of thymic stromal cells. For RTOC experiments, E14.5 embryos were obtained from time-mated C57BL6/N females. The morning of vaginal plug detection was taken as E0.5. NMRI *nude/nude* 6-week-old females were used for the isolation of thymic stromal cells and as hosts in RTOC transplantation experiments. *FoxN1*^{Cre} knockin mice were kindly provided by N. Manley (University of Georgia) (Foxn1tm3(cre)Nrm) (Liston et al., 2007). Rosa-Tomato-GFP animals (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J) (Muzumdar et al., 2007) were kindly provided by R. Sandhoff (DKFZ). CAG-GFP-tg animals were kindly provided by E. Erikci and K. Chowdhury (MPI for Biophysical Chemistry). β5T-Venus animals (B6-Psmb11tm1Smta) (Murata et al., 2007) were kindly provided by S. Murata (University of Tokyo). K5-H2B-GFP animals (Tg(KRT5-tTA)#Glk Tg(tetO-HIST1H2BJ/GFP)47Efu/J) (Tumbar et al., 2004) were kindly provided by P. Angel (DKFZ) and T. Barthlott (University of Basel). All animal experiments were approved by the regional authorities (Regierungspräsidium Karlsruhe, Germany) and performed according to the guidelines of the German Cancer Research Center (DKFZ).

Human thymi were obtained from male and female children under 3 years of age in the course of corrective cardiac surgery at the Department of Cardiac Surgery, Medical School of the University of Heidelberg, Germany. Studies on human samples were approved by the Institutional Review Board of the University of Heidelberg.

Thymic Stromal Cell Isolation and Sorting

Thymic stromal cell isolation from adult animals and human donors were performed as previously described (Ucar et al., 2013) with the modification of omitting FCS from RPMI medium. Embryonic thymi were digested with collagenase/dispase for 30 min at 37°C. Digested thymic tissue was depleted of CD45$^+$ cells using CD45 MicroBeads (Miltenyi Biotec). For detailed protocol, see Supplemental Experimental Procedures. For FACS sorting experiments, thymic stroma-enriched single-cell suspensions were blocked with anti-FcR mAb 2.4G2 and stained for various surface markers (see Supplemental Experimental Procedures, Antibodies). For dead cell exclusion, PI or DAPI were used. For intracellular staining (pan-CK), cells were fixed after surface marker staining in 1% PFA and permeabilized in 0.02% Triton X-100. Cell sorting and subsequent analysis was performed on a BD FACSAria cell sorter and FlowJo (Treestar), respectively. Live imaging of the thymosphere cultures was performed using a motorized inverted Cell Observer Z1 (Zeiss) and image analyses for the quantification of thymospheres were done using Axiovision software (Zeiss). For counting of thymospheres, we applied a diameter cut-off of minimum 75 × 65 μm for two perpendicular axes. In quantification experiments, we plated each sorted cellular fractions in 96-well plates using at least two different 10-fold dilutions (e.g., 10^5 cells/ml and 10^4 cells/ml) and in multiple (up to 8) technical replicates. To avoid false positives due to secondary aggregation during thymosphere formation in the prospectively enriched fractions (which should contain more TSFCs per well), we counted the lowest dilution plated that still contained at least one sphere per replicate (well).

Sphere Culture and PKH-26 Labeling

Spheres were grown in ultralow attachment plates (Corning) at variable densities in MEBM medium (Lonza) supplemented with B27 supplement (GIBCO), 0.5 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 4 μg/ml heparin (Sigma), 20 ng/ml bFGF (Invitrogen), and 20 ng/ml EGF (Sigma). For precise enumeration of thymospheres under various experimental conditions, we determined the optimal plating dilution to be 10^5 nonhematopoietic cells (NHC)/ml. Unless indicated otherwise, spheres were collected on day 7–8 of culture. PKH-26 labeling was performed on fresh thymic digests using PKH-26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma) at a concentration of 1×10^{-9} M PKH-26 dye per 1×10^5 cells according to the manufacturer's protocol. Imaging of spheres was performed with a motorized inverted Cell Observer Z1 (Zeiss).

For the sphere re-formation assay, thymospheres were collected by size selection on 35 μm filters, dissociated into single cells both enzymatically (10 min in Accumax, Sigma) and mechanically by gentle pipetting, filtered through 35 μm filters, and plated in the thymosphere-culturing medium as described above at a density of 10^4 and 10^3 cells per ml.

In Vitro Differentiation of Spheres

Spheres were size-selected on 35 μm filter, dissociated enzymatically (2 min in 0.05% trypsin, 0.53 mM EDTA-4Na; Invitrogen) and mechanically by gentle pipetting, and plated onto collagen-coated coverslips in DMEM/F12 supplemented with 10% FCS, 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 20 ng/ml bFGF (Invitrogen), 20 ng/ml EGF (Sigma), and Pen/Strep. After 9 days in culture, cells were fixed in 4% PFA in PBS, washed with PBS, permeabilized in 0.5% Triton X-100, and stained for TEC lineage markers in PBS/BSA (1% BSA in PBS). Cells were mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes). Confocal imaging was performed with an Olympus FluoView FW1000 motorized inverted microscope.

Whole-Mount Staining

Spheres were fixed in 2% paraformaldehyde, 100 mM HEPES, 50 mM EGTA, 10 mM $MgSO_4$, and 0.05% glutaraldehyde, washed in PBS/BSA, and permeabilized in 0.2% Triton X-100 in PBS/BSA overnight. After blocking in 3% BSA in PBS, spheres were stained at RT with antibodies against various markers (K8, K14, pan-CK, CD205, vimentin, CD31) in PBS/BSA and mounted with ProLong gold antifade reagent with DAPI (Molecular Probes). Confocal imaging was performed using an Olympus FluoView FW1000 motorized inverted microscope.

Reaggregate Thymic Organ Culture and Transplantation

Thymic stroma reaggregates were prepared from fetal thymic stromal cells as previously described (Jenkinson et al., 1992). In brief, thymi derived from E14.5 C57BL/6N embryos were digested with trypsin solution for 5 min at 37°C and mixed with thymospheres derived from Rosa-Tomato-GFP reporter mouse line. The reaggregates were placed on a 0.8 μm Nucleopore filter (Whatman) covering a floating sponge in a medium-filled 6-well plate. For long-term reconstitution assays, RTOCs were grafted under the kidney capsule of adult NMRI *nude* mice after 1 day of culture (Rodewald et al., 2001; Gill et al., 2002). The transplants were analyzed 4 and 6 weeks later by flow cytometry or histology.

Statistics

The stem cell frequency in the limiting dilution experiment was assessed using the ELDA (extreme limiting dilution analysis) software (http://bioinf.wehi.edu.au/software/elda/) (Hu and Smyth, 2009). A 95% confidence interval of active cell frequency was computed for two independent experiments, and single-hit hypothesis was tested by the software using the goodness-of-fit test.

(C) Representative pictures of thymosphere cultures of the respective sorted fractions. Scale bars represent 1 mm. Data are representative of three independent experiments.

(D) A thymosphere marked by the rectangle in (C) formed by the FoxN1$^-$ lineage fraction is shown in brightfield, green (for GFP expression), and red (for Tomato expression) fluorescence channels along with the corresponding overlay. Scale bar represents 100 μm.

(E) Representative confocal image planes of a thymosphere formed by thymic stromal cells of *nude* mice stained for K8 (cTEC) and K14 (mTEC) and counterstained with DAPI. Scale bar represents 20 μm.

(F) FoxN1 upregulation in monolayer attachment culture on day 12 of in vitro differentiation of cells derived from dissociated thymospheres (thymospheres were derived from sorted Tomato$^+$ cells of Rosa-Tomato-GFP × *Foxn1*^{Cre} animals). Scale bar represents 50 μm. Data are representative of three independent experiments.

See also Figure S5.

For the calculation of statistical significance in enrichment of TSFCs within different sorted cellular fractions, Student's t test method was used. p values smaller than 0.05 were considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.07.005.

AUTHOR CONTRIBUTIONS

A.U., O.U., and P.K. designed and performed the experiments and analyzed and interpreted results. S.M. performed all confocal analyses and contributed to the sphere re-formation assay. F.B. assisted during some transplantation experiments. T.G.H. and B.K. directed the study. A.U., O.U., T.G.H., and B.K. wrote the manuscript, and all authors read and approved the manuscript.

ACKNOWLEDGMENTS

We thank N. Manley, R. Sandhoff, E. Erikci, K. Chowdhury, S. Murata, P. Angel, and T. Barthlott for providing transgenic mice used in this study. We are grateful to the DKFZ Core Facilities Light Microscopy and Flow Cytometry for technical support. We are indebted to C. Sebening and S. Loukanova (Department of Cardiac Surgery, Medical School of the University of Heidelberg) for providing human thymic tissue. This study was supported by the DKFZ, the EU consortium "Tolerage" (B.K.), the European Research Council (ERC-2012-AdG to B.K.), the Baden-Württemberg Foundation (T.G.H.), and the German Federal Ministry of Education and Research (BMBF GerontoSysII Program to T.G.H.).

Received: April 30, 2014
Accepted: June 13, 2014
Published: August 21, 2014

REFERENCES

Anderson, G., and Takahama, Y. (2012). Thymic epithelial cells: working class heroes for T cell development and repertoire selection. Trends Immunol. *33*, 256–263.

Baik, S., Jenkinson, E.J., Lane, P.J., Anderson, G., and Jenkinson, W.E. (2013). Generation of both cortical and Aire(+) medullary thymic epithelial compartments from CD205(+) progenitors. Eur. J. Immunol. *43*, 589–594.

Bleul, C.C., Corbeaux, T., Reuter, A., Fisch, P., Mönting, J.S., and Boehm, T. (2006). Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature *441*, 992–996.

Boehm, T. (2008). Thymus development and function. Curr. Opin. Immunol. *20*, 178–184.

Bonfanti, P., Claudinot, S., Amici, A.W., Farley, A., Blackburn, C.C., and Barrandon, Y. (2010). Microenvironmental reprogramming of thymic epithelial cells to skin multipotent stem cells. Nature *466*, 978–982.

Chen, L., Xiao, S., and Manley, N.R. (2009). Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. Blood *113*, 567–574.

Cheng, L., Guo, J., Sun, L., Fu, J., Barnes, P.F., Metzger, D., Chambon, P., Oshima, R.G., Amagai, T., and Su, D.M. (2010). Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy. J. Biol. Chem. *285*, 5836–5847.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., and Wicha, M.S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev. *17*, 1253–1270.

Eirew, P., Stingl, J., Raouf, A., Turashvili, G., Aparicio, S., Emerman, J.T., and Eaves, C.J. (2008). A method for quantifying normal human mammary epithelial stem cells with in vivo regenerative ability. Nat. Med. *14*, 1384–1389.

Gäbler, J., Arnold, J., and Kyewski, B. (2007). Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. Eur. J. Immunol. *37*, 3363–3372.

Gill, J., Malin, M., Holländer, G.A., and Boyd, R. (2002). Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. Nat. Immunol. *3*, 635–642.

Gordon, J., and Manley, N.R. (2011). Mechanisms of thymus organogenesis and morphogenesis. Development *138*, 3865–3878.

Gordon, J., Bennett, A.R., Blackburn, C.C., and Manley, N.R. (2001). Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. Mech. Dev. *103*, 141–143.

Gray, D., Abramson, J., Benoist, C., and Mathis, D. (2007). Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J. Exp. Med. *204*, 2521–2528.

Hamazaki, Y., Fujita, H., Kobayashi, T., Choi, Y., Scott, H.S., Matsumoto, M., and Minato, N. (2007). Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. Nat. Immunol. *8*, 304–311.

Heng, T.S., Chidgey, A.P., and Boyd, R.L. (2010). Getting back at nature: understanding thymic development and overcoming its atrophy. Curr. Opin. Pharmacol. *10*, 425–433.

Hu, Y., and Smyth, G.K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods *347*, 70–78.

Jenkinson, E.J., Anderson, G., and Owen, J.J. (1992). Studies on T cell maturation on defined thymic stromal cell populations in vitro. J. Exp. Med. *176*, 845–853.

Klug, D.B., Carter, C., Crouch, E., Roop, D., Conti, C.J., and Richie, E.R. (1998). Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. Proc. Natl. Acad. Sci. USA *95*, 11822–11827.

Lim, E., Vaillant, F., Wu, D., Forrest, N.C., Pal, B., Hart, A.H., Asselin-Labat, M.L., Gyorki, D.E., Ward, T., Partanen, A., et al.; kConFab (2009). Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat. Med. *15*, 907–913.

Liston, A., Farr, A.G., Chen, Z., Benoist, C., Mathis, D., Manley, N.R., and Rudensky, A.Y. (2007). Lack of Foxp3 function and expression in the thymic epithelium. J. Exp. Med. *204*, 475–480.

Manley, N.R., and Condie, B.G. (2010). Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. Prog. Mol. Biol. Transl. Sci. *92*, 103–120.

Manley, N.R., Richie, E.R., Blackburn, C.C., Condie, B.G., and Sage, J. (2011). Structure and function of the thymic microenvironment. Front Biosci (Landmark Ed) *16*, 2461–2477.

Moll, R., Divo, M., and Langbein, L. (2008). The human keratins: biology and pathology. Histochem. Cell Biol. *129*, 705–733.

Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007). Regulation of CD8+ T cell development by thymus-specific proteasomes. Science *316*, 1349–1353.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis *45*, 593–605.

Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H., and Boehm, T. (1994). New member of the winged-helix protein family disrupted in mouse and rat nude mutations. Nature *372*, 103–107.

Nowell, C.S., Bredenkamp, N., Tetélin, S., Jin, X., Tischner, C., Vaidya, H., Sheridan, J.M., Stenhouse, F.H., Heussen, R., Smith, A.J., and Blackburn, C.C. (2011). Foxn1 regulates lineage progression in cortical and medullary thymic epithelial cells but is dispensable for medullary sublineage divergence. PLoS Genet. *7*, e1002348.

Ohigashi, I., Zuklys, S., Sakata, M., Mayer, C.E., Zhanybekova, S., Murata, S., Tanaka, K., Holländer, G.A., and Takahama, Y. (2013). Aire-expressing thymic medullary epithelial cells originate from β5t-expressing progenitor cells. Proc. Natl. Acad. Sci. USA *110*, 9885–9890.

Pastrana, E., Silva-Vargas, V., and Doetsch, F. (2011). Eyes wide open: a critical review of sphere-formation as an assay for stem cells. Cell Stem Cell *8*, 486–498.

Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., Bernard, L., Viale, G., Pelicci, P.G., and Di Fiore, P.P. (2010). Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell *140*, 62–73.

Reynolds, B.A., and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. *175*, 1–13.

Rodewald, H.R. (2008). Thymus organogenesis. Annu. Rev. Immunol. *26*, 355–388.

Rodewald, H.R., Paul, S., Haller, C., Bluethmann, H., and Blum, C. (2001). Thymus medulla consisting of epithelial islets each derived from a single progenitor. Nature *414*, 763–768.

Rossi, S.W., Jenkinson, W.E., Anderson, G., and Jenkinson, E.J. (2006). Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. Nature *441*, 988–991.

Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L., Lindeman, G.J., and Visvader, J.E. (2006). Generation of a functional mammary gland from a single stem cell. Nature *439*, 84–88.

Shakib, S., Desanti, G.E., Jenkinson, W.E., Parnell, S.M., Jenkinson, E.J., and Anderson, G. (2009). Checkpoints in the development of thymic cortical epithelial cells. J. Immunol. *182*, 130–137.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., and Eaves, C.J. (2006). Purification and unique properties of mammary epithelial stem cells. Nature *439*, 993–997.

Trzpis, M., McLaughlin, P.M., de Leij, L.M., and Harmsen, M.C. (2007). Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am. J. Pathol. *171*, 386–395.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359–363.

Tykocinski, L.O., Sinemus, A., and Kyewski, B. (2008). The thymus medulla slowly yields its secrets. Ann. N Y Acad. Sci. *1143*, 105–122.

Ucar, O., Tykocinski, L.O., Dooley, J., Liston, A., and Kyewski, B. (2013). An evolutionarily conserved mutual interdependence between Aire and microRNAs in promiscuous gene expression. Eur. J. Immunol. *43*, 1769–1778.

Wang, X., Laan, M., Bichele, R., Kisand, K., Scott, H.S., and Peterson, P. (2012). Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. Front. Immunol. *3*, 19.

Welm, B.E., Tepera, S.B., Venezia, T., Graubert, T.A., Rosen, J.M., and Goodell, M.A. (2002). Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev. Biol. *245*, 42–56.